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13. ABSTRACT (Maximum 200 Words)

The mammalian ErbB signaling network, composed of multiple polypeptide ligands belonging to the epidermal growth factor (EGF) superfamily, and four related type I receptor tyrosine kinases, EGFR/ErbB1-ErbB4, transduces both growth and differentiation signals in a variety of cell types. We have previously derived mice lacking EGF family growth factors, individually and in combination, to reveal roles in the mammary gland and gut. To investigate physiological roles for heparin-binding EGF (HB-EGF) we generated HB-EGF' mice by targeted mutation of the gene. Although no defects in mammary gland development or function were found, we did discover defects in the architecture of the lung and heart valves suggesting HB-EGF normally mediates cellular differentiation in these tissues. We also found the same heart valve defect in mice with a targeted mutation of the ADAM-family integral membrane metalloprotease, Tumor Necrosis Factor-Alpha Converting Enzyme (TACE) and mice lacking EGFR, suggesting that soluble HB-EGF is the critical EGFR-binding ligand regulating lung and cardiac valve maturation. Additionally, it appears that HB-EGF/EGFR signaling may modulate bone morphogenic protein signaling in heart valves.

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Training Grant Annual Report

INTRODUCTION

The mammalian ErbB signaling network is composed of multiple polypeptide ligands belonging to the epidermal growth factor (EGF) superfamily, and four related type I receptor tyrosine kinases, EGFR/ErbB1-ErbB4 (reviewed in 1). These members transduce both growth and differentiation signals in a variety of cell types and studies implicate this network in both physiological and pathological processes, including breast development and tumorigenesis. Animal models generated recently in our laboratory exposed specific roles for EGF ligands in the mammary gland (2) and gut (3). Thus, the goal of my research was to generate new mouse models for ErbB3 and ErbB4 in order to further analyze the role of the ErbB network in mammary gland development. To accomplish this I proposed to generate mammary gland specific knockout mice for ErbB3 and ErbB4, and to analyze ErbB3 expression and signaling in cultured mouse mammary epithelial cells. Additionally, in a continuing effort to identify physiological roles for EGFR-binding ligands, I characterized newly-derived gene targeted mice lacking either heparin-binding EGF (HB-EGF) or betacellulin (BTC). Both ligands are widely distributed in mammalian tissues including mammary gland (4), and both activate EGFR and ErbB4, albeit with different biological responses.

Although generation of mammary gland specific ErbB3 and ErbB4 knockout mice has not been completed, I have discovered that mice lacking HB-EGF exhibit impaired development of their cardiac valves and lungs (5). Also, mice lacking EGFR or TNF-Alpha Converting Enzyme (TACE) have the same heart and lung phenotypes suggesting that HB-EGF is the critical EGFR-binding ligand regulating lung and cardiac valve maturation and that soluble HB-EGF, resulting from TACE cleavage of membrane-bound HB-EGF, may be required for these processes. Finally, my research provides evidence that HB-EGF/EGFR signaling modulates TGF-β family signaling in cardiac valvulogenesis, supporting cell culture studies (6,7).

BODY

In order to generate mammary gland specific knockout mice for ErbB3 and ErbB4, genomic clones were isolated from a 129/sv mouse genomic library using mouse cDNA probes generated in the mentor's laboratory. Subclones were analyzed by restriction digest, PCR, and DNA sequencing, and construction of targeting vectors is underway.

Characterization and analysis of HB-EGF-null (HB-EGF') mice revealed that this ligand is required for heart and lung development, for targeted mice exhibit impaired lung branching morphogenesis, abnormal cardiac valvulogenesis, and cardiomegaly. Although mice lacking BTC do not exhibit an overt phenotype, mice lacking both HB-EGF and BTC exhibit increased rates of heart failure. Additionally, we discovered that mice lacking the HB-EGF receptor, EGFR, have the same lung and heart defects. I also examined hearts of transgenically rescued ErbB4 knockout mice (M. Gassmann, unpublished data), but did not find any heart valve defects, suggesting that this receptor is not critical for heart valve development.

Our recent investigations of the ADAM-family integral membrane metalloprotease, Tumor Necrosis Factor-Alpha Converting Enzyme (TACE) as a physiological convertase for TGFα and possibly other EGF family members led us to examine their hearts as well. TACE^{-/-} mice typically die around birth, displaying anomalies in the organization of various epithelia (8), but the exact cause of death is not known. I discovered that newborn TACE^{-/-} hearts also have similarly defective valves. Thus, our results identify HB-EGF as the critical EGFR-binding



ligand that regulates lung and cardiac valve maturation, and suggests that TACE processing of membrane-bound HB-EGF may be required for these processes.

Microarray analysis revealed that several genes implicated in bone morphogenic protein (BMP) signaling were upregulated in HB-EGF $^{\prime\prime}$ hearts during embryonic development. BMPs are members of the TGF- β superfamily that have substantial roles in heart development. Immunostaining hearts with an antibody that recognizes activated Smad signaling components verified this observation for HB-EGF $^{\prime\prime}$ valves showed excessive staining compared to controls.

Histological examination of lungs from HB-EGF^{-/-} newborns revealed that there were fewer alveoli, abnormally thickened mesenchymal tissue, and large dilated airways compared to controls. Additionally, reduced levels of prosurfactant C were identified by immunostaining (histological sections of HB-EGF^{-/-} lung.) Both EGFR^{-/-} and TACE --- mice exhibit similar defective lung branching and alveolar immaturity (9, 10).

KEY RESEARCH ACCOMPLISHMENTS

- Genomic clones isolated from a 129/sv mouse genomic library using mouse cDNA probes to ErbB3 and ErbB4 generated in the mentor's laboratory have been analyzed by restriction digest, PCR, and DNA sequencing, and construction of targeting vectors is underway.
- Histological examination of hearts from HB-EGF-null mice revealed that the atrioventricular (AV) and semilunar (SL) heart valves were abnormally large due to an overabundance of mesenchymal cushion cells.
- Approximately 50% of HB-EGF^{-/-} mice died perinatally. Echocardiography of surviving HB-EGF^{-/-} mice revealed cardiomegaly and impaired heart function. Adult mice also exhibiting ectopic cartilage formation in the valve leaflets.
- HB-EGF^{-/-}, EGFR^{-/-}, and TACE^{-/-} mice all have similarly defective AV and SL valves, unlike the EGFR^{wa2/wa2} mouse mutant, in which only the SL valves are enlarged.
- *in situ* hybridization analyses detected EGFR, ErbB4, ErbB3, and HB-EGF and TACE expression in the developing heart.
- Quantitative measurement of the aortic valves of newborn HB-EGF^{-/-}, EGFR^{-/-}, and TACE^{-/-} mice confirms that they are all significantly enlarged, relative to wild type valves.
- Measurement of endocardial cushions and valves during embryogenesis reveals that HB-EGF^{-/-} valves form normally but do not undergo remodeling during late gestation (E14.5-E15.5), as do HB-EGF wild type valves.
- Quantification of cells in endocardial cushions and valves at E14.5 and E15.5 via TUNEL assays and BrdU-incorporation reveals that excessive cell proliferation occurs in HB-EGF^{-/-} hearts in late gestation.
- Gene array analysis of RNA from embryonic hearts at 14.5 days of age identified three upregulated genes, dHAND, Smad6, and Id-1, all implicated in BMP signaling.
- Immunohistochemical staining with phospho-Smad 1/5/8 antibody revealed upregulated Smad signaling in HB-EGF^{-/-} valves during embryonic development.
- Lungs from HB-EGF^{-/-} newborns have abnormally thickened mesenchymal tissue, fewer alveoli, and reduced levels of surfactant protein as seen in EGFR^{-/-} and TACE ^{-/-} mice.

• BTC-null mice did not exhibit an overt phenotype, but combinatorial mouse mutants lacking both HB-EGF and BTC exhibit increased rates of heart failure compared to HB-EGF^{-/-} mice.

REPORTABLE OUTCOMES

All of these results have been recently published (see appendix A).

CONCLUSIONS

Heparin-binding epidermal growth factor (HB-EGF) is a ligand member of the ErbB family of proteins composed of multiple polypeptide ligands and four type I receptor tyrosine kinases. Numerous studies have confirmed that membrane-bound proHB-EGF is proteolytically cleaved to release soluble growth factor that binds EGFR and ErbB4 and stimulates cell growth in culture. However the protease(s) involved in shedding, the relevance of multiple receptors, and the specific functions of HB-EGF are still in question. My analysis of HB-EGF¹ mice revealed that it is required for normal development of the heart and lung and that EGFR¹ and TACE¹ newborn mice also have the same heart valve phenotype. The similarities in phenotypic defects suggests that HB-EGF is the critical EGFR-binding ligand regulating lung and cardiac valve maturation and suggests that soluble HB-EGF, resulting from TACE processing of membrane-bound HB-EGF, may be required for these processes. Additionally, it appears that defective valvulogenesis in HB-EGF¹ mice may result from a loss of BMP regulation. This is the first in vivo evidence that the EGFR signaling modulates TGF- β family signaling as suggested from culture experiments.

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APPENDICES

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Defective valvulogenesis in HB-EGF and TACE-null mice is associated with aberrant BMP signaling

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Heparin-binding epidermal growth factor (HB-EGF) and betacellulin (BTC) are activating ligands for EGF receptor (EGFR/ErbB1) and ErbB4. To identify their physiological functions, we disrupted mouse HB-EGF and BTC alleles by homologous recombination. Most HB-EGF-/- mice died before weaning, and survivors had enlarged, dysfunctional hearts and reduced lifespans. Although BTC-/- mice were viable and fertile and displayed no overt defects, the lifespan of double null HB-EGF-/-/BTC-/- mice was further reduced, apparently due to accelerated heart failure. HB-EGF-/newborns had enlarged and malformed semilunar and atrioventricular heart valves, and hypoplastic, poorly differentiated lungs. Defective cardiac valvulogenesis was the result of abnormal mesenchymal cell proliferation during remodeling, and was associated with dramatic increases in activated Smad1/5/8. Consistent with the phenotype, HB-EGF transcripts were localized to endocardial cells lining the margins of wildtype valves. Similarly defective valvulogenesis was observed in newborn mice lacking EGFR and tumor necrosis factor-a converting enzyme (TACE). These results suggest that cardiac valvulogenesis is dependent on EGFR activation by TACE-derived soluble HB-EGF, and that EGFR signaling is required to regulate bone morphogenetic protein signaling in this context.

Keywords: ADAM17/betacellulin knockout/heart failure/lung/Smad

Introduction

The epidermal growth factor (EGF)-ErbB signaling network includes multiple polypeptide ligands of the EGF/neuregulin superfamily, and four related tyrosine kinase receptors, epidermal growth factor receptor (EGFR)/ErbB1-ErbB4 (reviewed by Riese and Stern, 1998). EGF-like growth factors share a conserved receptor binding motif and soluble forms are usually derived from

bioactive, integral membrane precursors. Besides EGF, they include transforming growth factor α (TGF α), amphiregulin (AR), heparin-binding epidermal growth factor (HB-EGF), betacellulin (BTC), epiregulin and the recently described epigen (Strachan et al., 2001). These proteins all bind to EGFR/ErbB1, inducing receptor dimerization, tyrosine kinase activation and downstream signaling pathways controlling cell proliferation, differentiation, survival or motility. Heparin-binding EGF and BTC can also bind to ErbB4 (Riese et al., 1996a; Elenius et al., 1997). EGF family members vary in their ability to activate distinct ErbB heterodimers, and this may partly account for differences in their bioactivities in cell culture (Beerli and Hynes, 1996; Riese et al., 1996b; Graus-Porta et al., 1997).

Numerous actions are ascribed to the EGF family of growth factors, but their physiological functions are not well understood. Hence, our laboratory has derived mice lacking several family members individually or in combination. These studies have revealed specific roles for TGFa in patterning and function of hair follicles and migration of fetal eyelid epidermis (Luetteke et al., 1993), and for AR supported by EGF and TGFa in ductal morphogenesis in the developing mammary gland (Luetteke et al., 1999). Additionally, all three ligands are required for optimal neonatal growth, in part reflecting their roles in the mammary gland, as well as their requirement for normal development and cytoprotection of the gastrointestinal tract (Troyer et al., 2001). In contrast, EGFR-/- mice usually die in utero, although the timing is markedly affected by the genetic background (Miettinen et al., 1995; Sibilia and Wagner, 1995; Threadgill et al., 1995). However, more recent tetraploid aggregation experiments have allowed development to term (Sibilia et al., 1998). In comparison, the restricted or mild phenotypes of EGF family knockouts indicate that there is considerable redundancy or cooperativity in the action of these ErbB ligands.

Here we report the inactivation of mouse HB-EGF and BTC. HB-EGF was first identified as a heparin-binding mitogen for fibroblasts and smooth muscle cells that was secreted by human macrophages (Besner et al., 1990; Higashiyama et al., 1991). It is widely distributed in mammalian tissues, including skin, lung and heart (reviewed by Raab and Klagsbrun, 1997). Induced at sites of wounding, HB-EGF may also function in response to vascular injury since it is expressed in atherosclerotic plaques by aortic smooth muscle cells and other cell types (Raab and Klagsbrun, 1997), and its production by endothelial cells is induced by VEGF (Arkonac et al., 1998) and TNFα (Yoshizumi et al., 1992). Heparinbinding EGF is also implicated in uterine growth and blastocyst implantation (Kennedy et al., 1993; Das et al., 1904)

Heparin-binding EGF is derived from a bioactive, transmembrane precursor, proHB-EGF, which also functions as the diphtheria toxin receptor in primates (Naglich et al., 1992). ProHB-EGF interacts with tetraspanin and other membrane proteins, and may distinctively signal growth inhibition or apoptosis rather than the proliferative response induced by soluble HB-EGF (reviewed by Iwamoto and Mekada, 2000). In this light, the identity of the protease that regulates HB-EGF shedding is an important topic that has assumed additional significance because regulated release of soluble HB-EGF contributes to transactivation of EGFR by G-protein-coupled receptors (GPCR) (Prenzel et al., 1999). Several metalloproteases, including members of the A Disintegrin and Metalloprotease (ADAM) (Izumi et al., 1998; Asakura et al., 2002; Lemjabbar and Basbaum, 2002; Yan et al., 2002) and matrix metalloprotease (MMP) (Suzuki et al.. 1997: Bursi et al., 2002; Yu et al., 2002) families, have been identified as putative proHB-EGF convertases.

BTC was identified as a mitogen secreted by pancreatic β -cell insulinomas (Sasada et al., 1993; Shing et al., 1993) and it is implicated in the normal development and function of the pancreas (reviewed in Dunbar and Goddard, 2000). It is highly expressed in fetal pancreas, promotes islet growth (Demeterco et al., 2000) and coregulates differentiation of insulin-producing cells (Mashima et al., 1996, 1999). It also promotes β -cell neogenesis and improves glucose tolerance in a mouse model of diabetes (Yamamoto et al., 2000). However, BTC is also widely expressed and additional roles in blastocyst implantation (Das et al., 1997), gastrointestinal function (Kallincos et al., 2000) and atherosclerosis (Tamura et al., 2001) have been proposed.

We report here that HB-EGF-/- and BTC-/- mice are viable and fertile, but HB-EGF-/- mice have severely shortened lifespans and exhibit specific cardiac and lung defects. These phenotypes are mimicked in mice lacking either EGFR or tumor necrosis factor-α converting enzyme (TACE), providing genetic evidence that these proteins are the relevant ErbB receptor and HB-EGF convertase, respectively.

Results

HB-EGF-/- and BTC-/- mice are viable and fertile

Mouse HB-EGF and BTC were disrupted in embryonic stem cells by homologous recombination (Figure 1A). HB-EGF exons 1 and 2 were replaced with PGK-Neo, thus deleting the signal peptide and propeptide, and simultaneously inducing a frameshift to prevent translational read through. BTC was inactivated by eliminating exon 3, which encodes the first two loops of the EGF-like sequence that are critical for receptor binding. Lines of HB-EGF-/- and BTC-/- mice were established from targeted ES clones on a mixed C57BL/6J × 129 background, and the desired targeting events verified by Southern blots of genomic DNA (Figure 1B) and exonspecific PCR (Figure 1C). Northern blots confirmed the absence of the respective transcripts (Figure 1D).

The average litter size from intercrosses of HB- $EGF^{+/-}$ mice was 10.2 (n = 13 litters, 132 pups), a typical size for our colony. However, 12 neonates died in the first week after birth and genotyping of the remaining 120 pups at

weaning revealed a non-Mendelian distribution of genotypes: 28%+/+, 62%+/- and 11%-/-. Surviving HB-EGF-/mice grew normally, were fertile and matings between them produced litters of typical size at birth, suggesting there was little to no intrauterine loss of HB-EGF- mice. However, only 40% of the HB-EGF-/- offspring survived to weaning (n = 7 litters, 20/50 pups survived) compared with >80% of control pups (n=5 litters, 32/39 pups survived), reminiscent of the results of HB-EGF+/matings. Mammary gland morphology was normal in HB-EGF-/- dams, and there was no functional evidence of impaired lactation. Gastrointestinal tract architecture was also normal in HB-EGF-/- neonates, and the body weights of surviving HB-EGF-/- pups were within normal range from 1 to 3 weeks of age (data not shown). Thus, increased mortality of HB-EGF-/- mice was not ascribed to defects in nursing or nutrient absorption.

Matings between $BTC^{+/-}$ parents produced offspring in the expected Mendelian ratios ($18\%^{+/+}$, $56\%^{+/-}$, $26\%^{-/-}$; n=14 litters) at weaning and $BTC^{-/-}$ mice were viable and grew normally. Matings between homozygous $BTC^{-/-}$ mice also yielded normal sized litters. Since BTC is implicated in the growth and development of the pancreas (Dunbar and Goddard, 2000), we specifically examined this tissue. No morphological differences were observed in newborn pancreata, and adult $BTC^{+/+}$ and $BTC^{-/-}$ males responded similarly to a glucose challenge test (data not shown). Also, expression of insulin, glucagon, cytokeratin and amylase were normal in $BTC^{-/-}$ pancreata (P.Miettinen, personal communication).

Defective cardiac valvulogenesis in HB-EGF-/- mice Examination of newborn hearts revealed grossly enlarged and stenotic semilunar (SL) (aortic and pulmonary) and atrioventricular (AV) valves in all newborn HB-EGF-/- mice (n = 14) compared with HB-EGF-/+ mice (n = 10) (Figure 2A–F). For example, the mean area of HB-EGF-/- aortic valves (Figure 2M) was $56.7 \pm 17 \text{ mm}^2$ (n = 7) compared with $21.9 \pm 4.6 \text{ mm}^2$ (n = 5) for HB-EGF-/+ controls (P = 0.0025).

Enlarged SL, but not AV, valves were observed in waved-2 (EGFRwa-2/wa-2) mice, which encode a kinaseimpaired EGFR mutant, and in EGFR-/- mice (Chen et al., 2000). We examined EGFRwa-2/wa-2 hearts and confirmed that only SL valves were enlarged (data not shown). However, both SL and AV valves were dramatically enlarged in EGFR-/- hearts (Figure 2G-I). Thus, aortic valve area in four EGFR-- hearts was $56.6 \pm 15.4 \text{ mm}^2$ compared with 16.7 ± 4.5 mm² in four control hearts (P = 0.0286) (Figure 2M). We attribute the difference between the two models to residual signaling by the wa-2 receptor and a lower threshold requirement in SL valves. Abnormal valves were not observed in triple null mice lacking AR, EGF and TGFa (Chen et al., 2000), nor in BTC-/- hearts (data not shown). Collectively, these results indicate that HB-EGF is uniquely required as an EGFR ligand for cardiac valvulogenesis.

TACE-/- mice display identical heart valve defects ADAM17/TACE is required for conversion of membrane-anchored proTGFα to the soluble ligand (Peschon et al., 1998; Sunnarborg et al., 2002). TACE-/- mice displayed generalized epithelial defects reminiscent of those

observed in EGFR-/- animals, and we showed in cell-based and in vitro peptide assays that TACE promotes HB-EGF processing (Sunnarborg et al., 2002). To assess whether TACE functions as an HB-EGF convertase in vivo, we examined the heart valves of newborn TACE-null mice. Hearts from these mice had SL and AV valves that were similarly enlarged compared with HB-EGF-/- and EGFR-/- counterparts (Figure 2J-L), e.g. the mean area of TACE-/-

aortic valves was $57.5 \pm 29.4 \text{ mm}^2$ compared with $20.0 \pm 7.4 \text{ mm}^2$ for $TACE^{+/+}$ controls (n = 4 each genotype; P = 0.0079) (Figure 2M).

Highly localized expression of HB-EGF in valves We examined HB-EGF expression in control hearts during cardiac valve development, from E11.5 through E15.5.

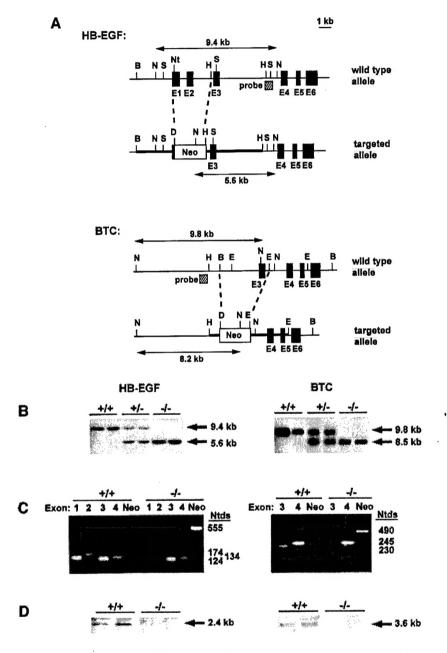
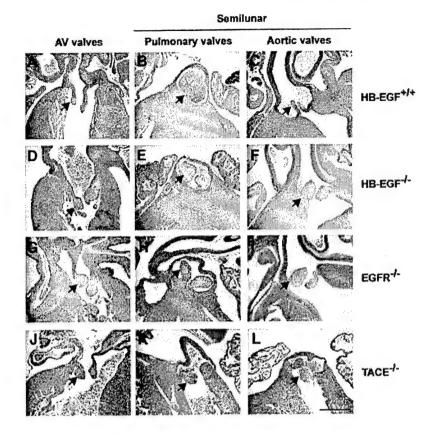
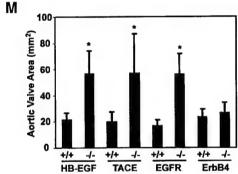


Fig. 1. Targeted disruption of HB-EGF and BTC. (A) Schematics illustrate strategies used to inactivate the mouse HB-EGF and BTC alleles. Targeting constructs were designed to eliminate exons (filled boxes) 1 and 2 of the HB-EGF gene, or exon 3 of the BTC gene using genomic sequences isolated from a mouse 129/Sv library. Heavy lines denote 5' and 3' arms of homology, and dashed lines bracket the genomic regions replaced by the Neo cassette. Hatched boxes mark probes used to verify correct targeting, and the locations of useful restriction sites and resulting fragments are noted. B, Bg/II; E, EcoRI; H, HindIII; N, NcoI; Nt, NorI; S, StuI. (B) Southern blot analyses of genomic DNAs digested with NcoI, showing results diagnostic for wild-type, heterozygous and homozygous targeted alleles using the indicated probes. (C) Genomic PCR using exon- or Neo-specific primers.

(D) Northern blots of lung RNA from mice of the designated genotypes. Arrows indicate the apparent mass of DNA or RNA species. Ntds, nucleotides; kb, kilobases.





HB-EGF transcripts were highly localized to the endocardial cells lining the margins of AV and SL (both aortic and pulmonary) valves, as shown at E14.5 (Figure 3A-F). Diffuse expression was also apparent throughout the myocardium, especially at later times (data not shown). EGFR transcripts were detected throughout the heart, including in developing valves, but enriched in the endocardial cushion, as shown for E14.5 (Figure 3G and H). TACE transcripts were found throughout the heart at this same developmental stage (Figure 3M and N), including in HB-EGF-expressing cells at the margins of AV and SL valves. Immunohistochemistry confirmed broad distribution of TACE protein (data not shown). Thus, growth factor, receptor and convertase are all coexpressed, consistent with the phenotypic similarities.

Since HB-EGF binds to ErbB4 as well as EGFR, we considered that ErbB4 might transduce HB-EGF signals required for valvulogenesis. ErbB4 transcripts were detected throughout the developing heart, including in cushions and valves, as shown at E14.5 in Figure 3K and L. This contrasts with restricted expression in the ventricular myocardium at E10.5 (Gassmann et al., 1995). Since ErbB4-/- embryos die early in gestation with defects in ventricular trabeculation, it was not possible to examine valvulogenesis in these mice. However, the knockout has recently been rescued by introduction of an ErbB4 transgene in heart muscle under the control of the α -myosin heavy chain promoter, which directs expression specifically to cardiomyocytes (Tidcombe et al., 2001). We examined SL and AV valves in these transgenic mice and found them to be of normal size (Figure 2M), suggesting that ErbB4 is not required for valvulogenesis. However, we cannot exclude possible low level expression of the transgene in valve mesenchyme sufficient to direct normal valvulogenesis.

We also observed *ErbB3* transcripts in the cushions and valves from E12.5 to 15.5 (Figure 3I and J), as previously shown for earlier time points (Meyer and Birchmeier, 1995). Thus, ErbB3 could potentially influence HB-EGF signaling through heterodimerization with EGFR.

Remodeling of valves requires HB-EGF

To address the mechanism of HB-EGF action, we compared valve development in HB-EGF-/-HB-EGF+/+ hearts from E12.5 to E15.5. Immature valves (AV and PV) were comparably sized at E12.5 and E13.5 in both genotypes, indicating that early events in valve formation occurred normally without HB-EGF. However, subsequent remodeling of valves was clearly perturbed. Whereas large, immature AV (Figure 4A and B) and PV (Figure 4E and F) valves of control hearts were remodeled to form slender valve leaflets, HB-EGF-/- valves remained enlarged throughout the period (Figure 4C, D, G and H). The largest size reduction for HB-EGF+/+ AV valves occurred from E13.5 to E14.5, and for PV valves from E12.5 to E13.5 (Figure 4Q and R). In contrast, HB-EGF-/valves were only modestly reduced in size, remaining 36-46% larger than the control counterparts through E15.5, differences that were significant (P = 0.0286) for AV valves at both time points and for PV valves at E15.5.

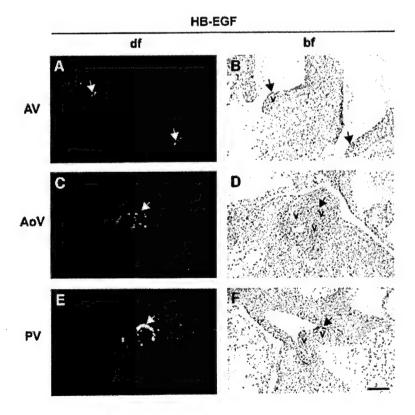
Impaired remodeling of *HB-EGF-* valves could be due to reduced cellular apoptosis or increased cellular proliferation. TUNEL assays revealed no differences in the

percentage of cells undergoing apoptosis in $HB\text{-}EGF^{+/+}$ versus $HB\text{-}EGF^{-/-}$ valves at E14.5 or E15.5 (Figure 4S). In contrast, the percentages of bromodeoxyuridine (BrdU)-positive cells were significantly (P = 0.0286) increased ~3.5-fold in AV valves and ~2-fold in PV valves of $HB\text{-}EGF^{-/-}$ hearts at E14.5 (Figure 4T). There was no change in the distribution of BrdU-positive cells in $HB\text{-}EGF^{-/-}$ valves, which were found throughout the valve mesenchyme. Thus, surprisingly, impaired remodeling of $HB\text{-}EGF^{-/-}$ valves was due to excessive proliferation of mesenchymal cells. Since the cardiac valves of young $HB\text{-}EGF^{-/-}$ adults were even larger and more disfigured than comparable controls (data not shown), we speculate that cellular proliferation continues beyond the embryonic time points analyzed here.

To determine whether the enlarged cardiac valves of newborn EGFR—and TACE—mice had a similar origin, we examined the respective hearts at E15.5. For both genotypes, AV and PV valves were significantly enlarged compared with controls, suggesting that impaired remodeling was also the cause of valve enlargement in these mutants (Figure 4I–P).

Bone morphogenetic protein (BMP) signaling is disregulated in the absence of HB-EGF

A clue to the mechanism of defective valvulogenesis came from an initial gene array comparison performed with total E14.5 HB-EGF+/+ and HB-EGF-/- hearts. The three genes most strongly upregulated in mutant heart, dHAND, Smad6 and Id-1 (data not shown), are all implicated in BMP signaling, which is required for outflow tract septation and valve development (Kim et al., 2001;



Gaussin et al., 2002; Delot et al., 2003). Since Smad6-/mice have hyperplastic valves (Galvin et al., 2000) similar to those observed here, presumably due to disregulated BMP signaling in the absence of this inhibitor Smad, and cell culture studies have established the potential for EGFR inhibition of BMP signaling (Kretzschmar et al., 1997; Nonaka et al., 1999), we speculated that defective valvulogenesis in our mice could be due to loss of BMP regulation. To test this, we examined activation of Smad 1/ 5/8 in sections of HB-EGF+/+ and HB-EGF-/- hearts using an antibody that detects phosphorylated forms of these BMP signaling effectors. Figure 5 reveals dramatically higher numbers of cells staining positive for nuclear phospho-Smad1/5/8 in pulmonary and aortic valves of mutant hearts compared with the control tissues at E14.5. A similar, albeit less dramatic, trend was seen in AV valves (data not shown). In contrast, HB-EGF+/+ and HB-EGF-/- valves showed similarly high levels of activated Smad1/5/8 in AV and aortic valves at E12.5 during the proliferative phase of valve development (data not shown).

HB-EGF-/- hearts are enlarged and dysfunctional

An unusually high proportion (43%) of HB-EGF-/- adult males and females died within the first year of life. At necropsy, these mice were found to have enlarged hearts and congested lungs, with no other apparent pathological defects. A systematic comparison showed that the heartto-body weight ratios of male HB-EGF-/- mice increased continuously from birth through 6 months of age compared with wild-type controls, although the largest relative increase occurred between 3 and 6 weeks of age (data not shown). Examples of enlarged hearts of apparently healthy, 6-week-old male HB-EGF-/- mice are shown in Figure 6A-C. The mean heart-to-body weight ratio for 6-week-old HB-EGF-/- mice was $1.27 \pm 0.20\%$ compared with 0.80 \pm 0.03% for HB-EGF^{+/+} controls (n = 5 both genotypes; P = 0.0079). At this stage, ventricular myocyte size (Figure 6D and E) and number per unit area were not significantly different between the two genotypes, indicating that enlargement was due to hypercellularity. However, we did not observe significant incorporation of BrdU into 5-week-old hearts of either genotype, suggest-

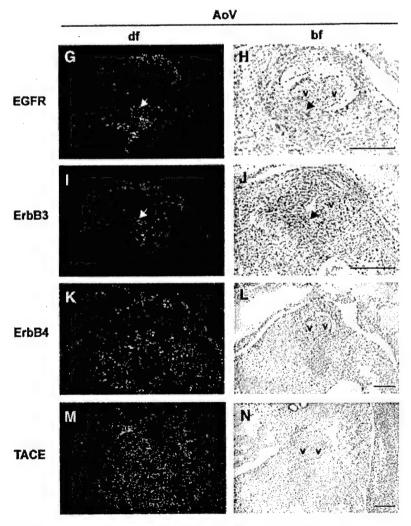


Fig. 3. In situ detection of HB-EGF, EGFR, ErbB3, ErbB4 and TACE transcripts in wild-type cardiac valves. (A-F) HB-EGF; (G and H) EGFR; (I and J)E rbB3; (K and L) ErbB4; (M and N) TACE. Arrows mark regions of high expression. Sense riboprobes were hybridized to confirm specific hybridization in each case (data not shown). AoV, aortic valve; bf, bright field; df, dark field; v, valves. Scale bars = 100 μm.

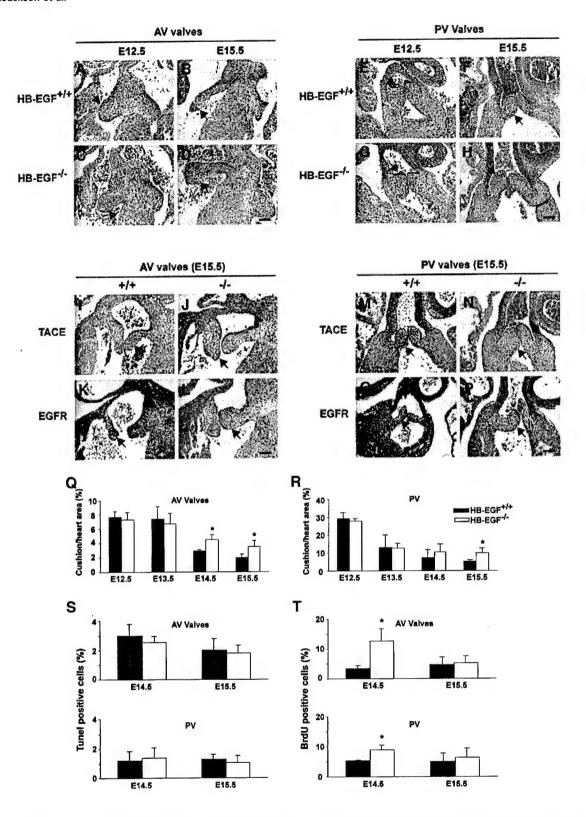


Fig. 4. Defective remodeling of cardiac valves in mutant mouse hearts. (A–H) H&E sections of $HB-EGF^{+/+}$ or $HB-EGF^{-/-}$ hearts showing (A–D) atrioventricular (AV) valves or (E–H) pulmonary valves (PV) at the indicated time points. (I–P) H&E sections of heart showing AV valves or PV valves of the indicated TACE or EGFR genotypes at E15.5. Arrows denote valves. Scale bars = 100 μ m. (Q–T) Quantitative comparison of valvulogenesis in $HB-EGF^{+/+}$ or $HB-EGF^{-/-}$ hearts. (Q and R) Cushion/heart ratio \pm SD at the indicated ages. (S) Percentage of TUNEL-positive cells at the indicated ages in AV valves or PV; (T) percentage of BrdU-positive cells. Filled bars, $HB-EGF^{+/+}$; unfilled bars, $HB-EGF^{-/-}$. Asterisks denote statistically significant results.

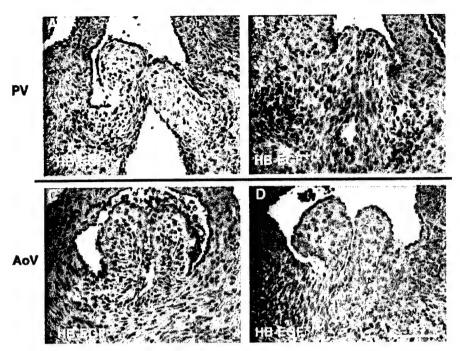


Fig. 5. Disregulated Smad signaling in HB-EGF mutant cushions. Sections of (A and B) pulmonary valve (PV) or (C and D) aortic valve (AoV) from E14.5 immunostained with phospho-Smad1/5/8 antibody and counterstained with hematoxylin. Arrows mark positive staining cells. Scale bar = 100 µM.

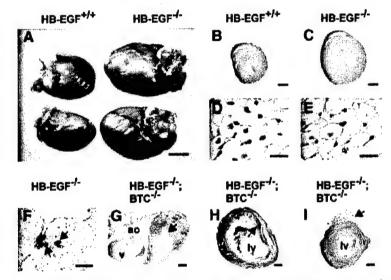


Fig. 6. Cardiac defects in HB-EGF^{+-/-} and HB-EGF^{+-/-} mice. (A) Hearts from 6-week-old male mice. (B and C) H&E-stained cross-sections of 6-week-old (B) HB-EGF^{+-/-} and (C) HB-EGF^{-/-/-} hearts. (D and E) PAS-stained transverse sections of left ventricle cardiomyocytes. (F) PAS-stained section of thickened aortic valve leaflet from 6-month-old HB-EGF^{-/-/-} heart. White arrow marks chondrocytes; black arrows denote ectopic cartilage formation. (G) H&E-stained section of HB-EGF^{-/-/-}/BTC^{-/-/-} heart. Arrow marks a thrombus in the left atrium. (H and I) Masson trichrome-stained sections of HB-EGF^{-/-/-}/BTC^{-/-/-} hearts. Arrows mark extensive intraventricular (H) or focal epicardial (I) fibrosis (blue stain) within or on the left ventricle, v, ventricle; ao, aorta; lv, left ventricle. Scale bars = 3.5 mm (A), 1 mm (B and C), 20 μm (D and E), 100 μm (F), 1 mm (G-I).

ing that proliferation occurred gradually. A comparison of transverse sections from 6-month-old male mice indicated that further heart enlargement included a significant hypertrophic component.

To assess the functionality of *HB-EGF*-/- hearts, we performed non-invasive transthoracic echocardiography (ECG) on 5-month-old mice. Left ventricular end-diastolic and -systolic dimensions were significantly larger, confirming cardiomegaly (Table I). Calculations of fractional

shortening (SF%) revealed significant systolic dysfunction in HB-EGF-- mice, which may be observed in advanced human aortic valve disease (although a myopathic contribution to the ECG observations cannot be excluded). Aortic dilation, a classic symptom of valvular stenosis, was also observed in ECGs (data not shown).

Histological sections of hearts from similarly aged HB-EGF--- mice showed grossly enlarged and deformed valves, and confirmed aortic dilation. Chondrocytes and

Table I. Echocardiogram results for HB-EGF and HB-EGF/BTC mice

Genotype	BW (g)	HR (b.p.m.)	IVST (mm)	LVEDD (mm)	LVESD (mm)	PWT (mm)	SF (%)
HB-EGF+/+	38.4 ± 3.9	659 ± 55	0.08 ± 0.03	0.32 ± 0.03	0.10 ± 0.02	0.1 ± 0.01	68.7 ± 2.7
HB-EGF-/-	35.3 ± 2.3	668 ± 21	0.10 ± 0.03	0.41 ± 0.05*	0.20 ± 0.05*	0.1 ± 0.02	51.6 ± 7.4*
HB-EGF+/+/BTC+/+	33.6 ± 2.8	653 ± 55	0.07 ± 0.01	0.30 ± 0.02	0.08 ± 0.01	0.07 ± 0.01	71.8 ± 3.3
HB-EGF-/-/BTC-/-	29.0 ± 2.5*	695 ± 43	0.07 ± 0.01	0.39 ± 0.08*	0.17 ± 0.10*	0.09 ± 0.01	58.8 ± 15.9*

Non-invasive transthoracic echocardiography was performed on unanesthetized, male mice. Values are mean \pm SD for 5-month-old HB-EGF+/+ (n = 5) and HB-EGF-/- (n = 5) mice, and 4-month-old HB-EGF+/+/BTC+/+ (n = 6) and HB-EGF-/-/BTC-/- (n = 6) mice. BW, body weight; HR, heart rate; IVST, interventricular septal distance; LVEDD, left ventricular end-diastolic dimension; LVESD, left ventricular end-systolic dimension; PWT, posterior wall thickness; SF, fractional shortening. Asterisks denote values significantly different from respective wild-type controls (P < 0.05).

ectopic cartilage formation were observed throughout the aortic valve leaflets of *HB-EGF*— but not control hearts (Figure 6F), possibly as a consequence of disregulated BMP signaling in *HB-EGF*— hearts. Cartilaginous metaplasia and ossification within the outflow tracts of the heart were observed in *Smad6*— mice (Galvin *et al.*, 2000), and in the cardiac valves of patients undergoing valve replacement (Mohler *et al.*, 2001).

Combined loss of BTC exacerbates heart defects

We interbred HB-EGF-/- and BTC-/- mice to generate double nulls in order to explore redundancy or cooperativity between these two EGFR/ErbB4 binding ligands. HB-EGF-/-/BTC-/- double null mice were viable and fertile, but displayed a significantly higher rate of mortality. Most (54%) were dead by 4 months of age, and 83% (n = 24) had died within the first year. As expected, cardiac valves of HB-EGF-/-/BTC-/- newborns were enlarged; however, the mean aortic valve area of newborn $HB-EGF^{-/-}/BTC^{-/-}$ mice (55.8 ± 8.4 mm²) was not significantly different from that of HB-EGF-/- mice (compare with values in Figure 2M). Electrocardiograms of 4-month-old HB-EGF-/-/BTC-/- mice confirmed cardiomegaly and systolic dysfunction compared with agematched controls (Table I). However, adult HB-EGF-/-/ BTC-/- mice displayed certain cardiac defects rarely or never seen in HB-EGF-/- mice. Left atrial dilation and thrombosis (see the clot in Figure 6G), as well as marked fibrosis within and on the left ventricle near the apex were commonly observed ante mortem in moribund HB-EGF-/-/BTC-/- mice from 2 months of age (Figure 6H) and I). We speculate that surface fibrosis may have resulted from friction between the HB-EGF-/-/BTC-/heart and the pericardium, possibly due to ventricular dilation (Figure 6H) during end-stage heart failure. None of these defects was observed in BTC-/- hearts. Ectopic cartilage formation observed in HB-EGF-/- hearts was also observed in HB-EGF-/-/BTC-/- mice, but not in BTC-/hearts (data not shown). Double null mice lacking HB-EGF and AR were also generated, but did not display aggravated heart defects or reduced lifespans relative to HB-EGF-/- mice (data not shown).

Morphological defects and alveolar immaturity in HB-EGF-/- lungs

We observed defects in the lungs in ~50% of HB-EGF—newborns. Mesenchymal tissue between alveoli was abnormally thickened compared with wild-type tissue (Figure 7A and B), and HB-EGF—lungs (n = 9) contained

significantly fewer alveoli than HB- $EGF^{+/+}$ tissue (n = 6): 39.2 ± 4.0 versus 28.1 ± 6.7 per field (P = 0.0012)(Figure 7G). These defects, which are consistent with HB-EGF expression in this tissue, were most striking in pups that were clearly in distress (no milk in the stomach, little movement), suggesting they may have contributed to the mortality of HB-EGF-/- pups. PAS staining revealed a much higher fraction of immature, glycogen-containing cells in newborn HB-EGF-/- lungs compared with controls (Figure 7C and D); conversely, fewer cells in HB-EGF-/lungs stained positive for the type II pneumocyte marker, pro-surfactant C (Figure 7E and F). Similar defects have been noted in EGFR-/- (Miettinen et al., 1995, 1997) and TACE-- lungs (Zhao et al., 2001), providing further evidence of a link between TACE and HB-EGF-EGFR signaling.

Discussion

Human heart valve defects are the most common type of congenital heart disease (CHD) (Bartram et al., 2001). Heart valves develop from endocardial cushions, which form when endothelial cells in the AV and outflow tract areas undergo an epithelial-to-mesenchymal transformation and invade the adjacent acellular matrix. The cells then proliferate to form cushions that subsequently give rise to the septa of the four-chambered heart as well as the cardiac valves (Eisenberg and Markwald, 1995; Lamers et al., 1995). In the mouse, endocardial cushion formation is complete by E12.5 (Lakkis and Epstein, 1998) and is followed by cellular apoptosis and differentiation, which remodel the cushions to slender valve leaflets. In mice lacking either HB-EGF or EGFR, valvulogenesis is disrupted, leading to thickened, stenotic valves. Thus, our findings corroborate a role for EGFR in valvulogenesis (Chen et al., 2000), extend this requirement to AV valves and establish that HB-EGF is uniquely required to activate EGFR ligand in this context.

Valvulogenesis is a complex process (Srivastava and Olson, 2000), and hence we considered various possible explanations for the *HB-EGF*— phenotype, including altered homing or migration of neural crest-derived cells. However, it is unlikely that the phenotype is due to aberrant neural crest cell migration since cushion formation, which also depends on this process, occurred normally. In addition, defects involving neural crest cells are typically associated with cardiac septal defects (Chien, 2000), and these are not observed in our mice. Finally, recent evidence suggests that neural crest cells do not

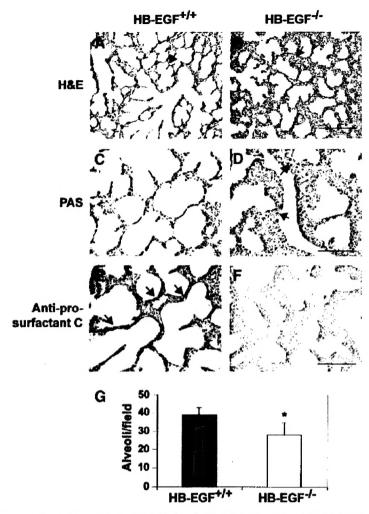


Fig. 7. A comparison of lung morphology and maturity in $HB-EGF^{+/+}$ and $HB-EGF^{-/-}$ mice. (A and B) H&E-stained sections of newborn lungs. Arrows point to thickened mesenchyme. (C and D) PAS-stained sections of newborn lung. Arrows (D) point to areas enriched in cells with high glycogen content. (E and F) Sections of newborn lung immunostained for prosurfactant C. Arrows point to positive cells. (G) Bars indicate mean number of alveoli counted from duplicate histological sections of newborn lung (n = 6-9 mice) of the indicated genotypes \pm SD. Asterisk denotes significant result (P = 0.0012). Scale bars = 200 μ m (A and B), 100 μ m (C-F).

contribute to AV valves (Jiang et al., 2000), which are similarly enlarged in our mice. Alternatively, we speculated that loss of the HB-EGF precursor, which selectively induced cellular apoptosis rather than proliferation in cell culture (Iwamoto et al., 1999), might reduce or eliminate cell death required for remodeling of the cardiac valves. However, the rate of apoptosis was unchanged in HB-EGF-/- valves during remodeling. Instead, our results indicate that the phenotype is due to excessive or unscheduled proliferation in the absence of HB-EGF, a surprising finding.

A possible clue to the mechanism of HB-EGF/EGFR action came from substantial evidence that cardiac valve formation depends on signaling by BMPs, members of the TGFβ superfamily. For example, mice encoding a genetically modified BMP2 receptor (BMP2R) with reduced signaling capacity displayed conspicuous defects in the outflow tract of the heart. These included a lack of conotruncal septation below the valves, interruption of the

aortic arch and a deficiency of SL valves (Delot et al., 2003). Similarly, double null mice lacking both BMP6 and BMP7, as well as mice deficient in Tolloid, a homolog of the Drosophila metalloprotease that cleaves the BMP antagonist, Chordin, also had hypoplastic valves (Clark et al., 1999; Kim et al., 2001). On the other hand, mice lacking the BMP signaling inhibitor, Smad6, had enlarged or hyperplastic valves (Galvin et al., 2000), indicating that Smad6 may regulate BMP2R signaling required for cardiac valvulogenesis. These various results suggest a model in which valves fail to form with insufficient BMP signaling, but are hyperplastic when BMP signaling is unregulated. The enlarged valves of EGFR-- and HB-EGF-/- mice are consistent with the hypothesis that EGFR, activated by HB-EGF, acts to downregulate BMP signaling in the developing valves. In the absence of either the ligand or its receptor, excessive BMP signaling leads to hyperproliferation. This hypothesis, which is consistent with the ability of EGFR to regulate BMP signaling in cell

culture (Kretzschmar et al., 1997; Nonaka et al., 1999), in part through phosphorylation and stabilization of the Smad transcriptional co-repressor TGIF (Lo et al., 2001), is supported by dramatic increases in cells staining positive for nuclear Smad1/5/8 in the hyperplastic HB-EGF-/- valves. To our knowledge, this is the first genetic evidence corroborating a physiological role for mammalian EGFR in transmodulating signaling by TGFβ superfamily members.

The similarities in heart as well as lung defects displayed by HB-EGF-/- and TACE-/- lines of mice strongly suggest that in addition to being a proTGFa convertase (Peschon et al., 1998; Sunnarborg et al., 2002), TACE is also a key regulator of HB-EGF shedding. The corollary of this conclusion is that soluble HB-EGF, not its integral membrane precursor, is required for normal valvulogenesis, although available reagents have not allowed us to confirm impaired shedding of endogenous HB-EGF from TACE-/- cells. However, recombinant TACE correctly cleaved a peptide substrate corresponding to the membrane-proximal cleavage site of proHB-EGF, and cotransfection of TACE enhanced the shedding of exogenous HB-EGF from TACE-/- fibroblasts (Sunnarborg et al., 2002). Hence, the combination of genetic, biochemical and cell biological evidence makes a compelling case for TACE as a physiological HB-EGF convertase. Other metalloproteases put forward as candidate HB-EGF convertases include MMP-3 (Suzuki et al., 1997; Bursi et al., 2002) and MMP-7/matrilysin (Yu et al., 2002), as well as the disintegrin metalloproteases ADAM9 (Izumi et al., 1998), ADAM10 (Lemjabbar and Basbaum, 2002; Yan et al., 2002) and ADAM12/meltrin α (Asakura et al., 2002). In particular, it was recently reported that GPCRactivated shedding of HB-EGF was blocked by a dominant-negative form of ADAM12, and that a metalloprotease inhibitor of HB-EGF shedding bound ADAM12 and inhibited hypertrophic changes in a mouse model (Asakura et al., 2002). However, although 30% of ADAM12-/- mice died prior to weaning, no heart defects were observed (Kurisaki et al., 2003). Conceivably, ADAM12 may have a role in induced but not constitutive shedding of HB-EGF.

Based on published reports as well as the findings reported here, it appears that all four ErbB receptors are required for normal heart development. Genetic evidence suggests that ErbB2 and ErbB4 form a NRG-1-activated heterodimer necessary for ventricular trabeculation (Gassmann et al., 1995; Lee et al., 1995; Erickson et al., 1997), and mice lacking any of these proteins die by E11.5. ErbB3, on the other hand, is essential for the formation of endocardial cushions, and ErbB3-/- mice frequently die in mid-gestation with abnormally small cushions (Erickson et al., 1997). In contrast, HB-EGF induced signaling by EGFR is required at a later stage for remodeling of the cardiac valves. The requirement for these related receptors at distinct stages of heart development is consistent with their observed ability to differentially activate various signaling pathways. An important, unanswered question is whether EGFR partners with another ErbB receptor to regulate valvulogenesis. The lack of valve defects in rescued ErbB4 hearts suggests that ErbB4 is not essential. The underdeveloped cushions of ErbB3-- hearts, on the other hand, indicate that this receptor is required for an early step in valvulogenesis (Camenisch et al., 2002), but do not exclude a requirement for ErbB3, possibly in partnership with EGFR, in the later stage of valve remodeling. Future studies should be aimed at determining whether EGFR functions as a homoor heterodimer in this context, as well as deciphering the respective mechanisms by which the four ErbB proteins regulate distinct stages of heart development.

Materials and methods

Reagents

B6EiC3H-a/A-EGFR^{wa-2/wa-2}Wnt3a^{vt} mice were from Jackson Laboratory (Bar Harbor, ME). EGFR-f- mice on a CD-1 or CD-1 × ALR.129 hybrid background were kindly provided by David Threadgill [University of North Carolina, Chapel Hill (UNC-CH)]. TACE*f- mice harboring the inactivating TACE^{ΔZn} mutation (Peschon et al., 1998), a gift from Jacques Peschon and Roy Black (Immunex, Seattle, WA), were maintained on a C57BL/6J × 129/Sv hybrid background. Hearts from transgene rescued ErbB4-f- mice were kindly provided by Martin Gassmann (National Institute for Medical Research, London).

Gene targeting

HB-EGF and BTC genomic clones were isolated from a 129/Sv mouse lambda library (Stratagene, La Jolla, CA) using a mouse HB-EGF cDNA (kindly provided by Michael Klagsbrun, Harvard Medical School, Boston, MA) or BTC cDNA. Plasmid pNTK was used to construct targeting vectors as depicted in Figure 1; resulting constructs were electroporated into R1 ES cells (Luetteke et al., 1999). Correctly targeted, G418-resistant ES cell clones of normal karyotype were microinjected into 3.5-day-old C57BL/6J blastocysts, which were then implanted into pseudopregnant CD-1 foster mothers by the UNC-CH Animal Models Core. Chimeras transmitting to germline were crossed to C57BL/6J partners, and subsequent generations of mice maintained on a mixed C57BL/6J × 129/Sv background. PCR primer sequences used for genotyping are available upon request.

Organ harvest and analysis

Neonatal mice were killed by halothane inhalation; older mice were asphyxiated by CO₂ according to IACUC guidelines. Excised organs were fixed in 10% buffered formalin for 48–72 h prior to paraffin embedding. Histological sections (5 µm) were prepared by the UNC-CH Histopathology Core and stained as indicated. Antibodies for immunohistochemistry were as follows: anti-Ki67 (sc-7846), Santa Cruz Biotechnology, Santa Cruz, CA; anti-phospho-Smad1(Scr463/465)/Smad5(Scr463/465)/Smad8(Scr462/428) (9511), Cell Signaling Technology, Beverly, MA; and anti-prosurfactant protein C (SURFCPabr), Research Diagnostics, Flanders, NJ. Bound antibodies were visualized with the horseradish peroxidase-based ABC Staining System kit (Santa Cruz Biotechnology, Santa Cruz, CA) (Schroeder and Lee, 1998). Negative controls were obtained by incubating primary antibodies with excess peptide immunogens prior to immunostaining.

For analysis of heart development, timed matings were performed, with E0.5 defined as the morning of the day that the vaginal plug was first observed. At select gestational ages, dams were killed, and embryos removed and fixed in 4% paraformaldehyde for 48 h prior to paraffin embedding. Measurements of endocardial cushions and valves were performed on captured hematoxylin-eosin (H&E) images using the Bioquant software program in the UNC-CH Imaging Core (supported by NCI grant P30-CA16086). To assess cell proliferation, pregnant dams were injected with 100 µg/g body weight BrdU (Cell Proliferation Labeling Reagent, Amersham Biosciences Corp., Piscataway, NJ) 2 h prior to death. Heart sections were then stained with mouse BrdU monoclonal antibody (Dako, Carpinteria, CA). To assess apoptosis, TUNEL assays were performed on heart sections using the ApopTag Plus Peroxidase in situ Apoptosis Detection kit (Intergen, Purchase, NY). The ratios of BrdU- and TUNEL-positive cells to total cells in heart cushions were determined for two sections per sample and averaged for statistical analyses.

RNA analyses

RNA was isolated for northern blots using Trizol as directed (Invitrogen, Carlsbad, CA). Samples were probed (Luetteke et al., 1999) using cloned HB-EGF or BTC cDNAs. In situ hybridization analyses were performed

on paraffin sections using cDNA-derived antisense riboprobes as described (Luetteke *et al.*, 1999; Kornblum *et al.*, 2000). Sense riboprobes were hybridized for controls.

Echocardiography

Two-dimensional and M-mode imaging were performed using an ATL HDI 5000 ultrasound system equipped with a CL10-5 CVasc/mouse scan head (Philips Medical Systems, Andover, MA). Left ventricular end diastolic dimension (*LVEDD*) and end systolic dimension (*LVESD*), interventricular septum thickness (*IVST*) and posterior wall thickness (*PWT*) were obtained from M-mode tracings. All LV dimension data are presented as the average of two measurements. The percentage of fractional shortening (%SF) was calculated from the equation:

 $%SF = (LVEDD - LVESD)/LVEDD \times 100.$

Statistical analysis

Statistical analyses were performed by UNC Lineberger Comprehensive Cancer Center Biostatistics Core using SAS statistical software (Version 8.2; SAS Institute, Cary, NC). Exact P values were calculated for all two-group comparisons using the non-parametric Wilcoxon two-group test.

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